OPTIMISATION AND COMPARISON OF THE STANDARD PCR AND THE REAL-TIME SYBR-GREEN HRM PCR FOR DETECTION OF CAV-2

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Abstract. The aim of this study was to optimise and compare the standard PCR method and the Real-Time Sybr-Green HRM PCR for detection of CAV-2. Studies were conducted on 20 CAV-2 vaccine isolates. DNA for molecular studies was isolated using a commercial isolation kit and a manual method with the use of phenol and chloroform. Due to the selection of suitable thermal conditions for individual reaction cycles and suitable reagent concentrations, both the PCR and the Real-Time PCR proved to be sensitive and specific techniques for detection of CAV-2 genetic material. The Real-Time PCR proved to be less time-consuming than the standard PCR, which is important from the clinical point of view. The performance of both reactions depended to a high extent on the DNA isolation method (the manual method showed a three times higher performance than the commercial kit). Presented results of the studies show that both the PCR and the Real-Time PCR are on target to become standard diagnostic methods for CAV-2 infections.

Keywords: CAV-2, dog, PCR, Real-Time PCR, molecular diagnosis.

Introduction

Adenoviruses are pathogens isolated from humans, as well as from farm and domestic animals (Buonavoglia and Martella, 2007; Koncicki et al., 2006; Lynch J.P. 3rd et al., 2011; Truszczyński and Pejsak, 2012; Wieliczko et al., 2003; Wiśniewski, 2004). Canine adenoviruses (CAVs) belong to the *Adenoviridae* family, the *Mastadenovirus* genus. They are non-enveloped viruses sized 70-90 nm, whose genome, in the form of a double DNA strand, is built of 36-44 kilobase pairs (Appel and Binn, 1987). Currently two antigen types of adenoviruses isolated from dogs are recognised: CAV-1, inducing infectious canine hepatitis (Rubarth's disease), and CAV-2, being an aetiological factor of canine tracheobronchitis (kennel cough) (Chaturved et al., 2008; Kalinowski et al., 2012). The total analysis of the genome of those adenoviruses showed their homology only in 75% (Davison et al., 2003; Morrison et al., 1997).

The following methods and techniques are used for the detection of CAV infections in dogs: cell culture studies, serological studies, electron microscopy and, recently, molecular techniques (PCR, Real-Time PCR) (Greene, 2012; Mizak and Rzeżutka, 1998; Mizak and Rzeżutka, 1997).

To achieve the highest possible sensitivity of the molecular study (to obtain the highest possible number of copies of the amplified gene fragment), it is necessary to select the optimal concentrations of substances comprising the reaction mixture, the reaction parameters and the base sequence (a proper gene selection), based on which PCR products will be amplified (Chesters, 1996; Chou, 1991; Kamińska and Dąbrowska, 2004).

The CAV-2 genome contains genes encoding numerous proteins that have specific functions during infection of the host's cells. The most important ones are: E1A and E1B (19K and 55K), E3, E4, pIVa2, pIIIa, pVI, pVII, pVIII, Pol, pTP, DBP. Out of the listed genes, E1B 19K could be used to detect CAV-2 in biological material. It is in charge of expression of a protein of the same name that is responsible for inhibition of apoptosis of the host's cells (White et al., 1988).

So far in Poland, as well as in eastern Europe, no extensive research into the epidemiology of CAV 2 infections has been carried out The only report on this topic indicates that the virus can be demonstrated in this part of world in 80% of dogs with symptoms of cough (Kalinowski et al., 2012). To be able to carry out large-scale epidemiological studies it is necessary to use a sensitive and rapid diagnostic test that allows the identification of the virus.

The aim of this study was to optimise and compare the traditional PCR method and the Real-Time Sybr-Green High Resolution Melting (HRM) PCR method for detection of CAV-2.

Materials and Methods

Studies were conducted on 20 vaccine CAV-2 isolates (strain Manhattan) obtained from two commercially available combined preparations used to immunize dogs against distemper, canine parvovirus and CAV infections (Nobivac DHP Intervet, Eurican DHPPi, Merial). Lyophilised vaccines were suspended in 0.5 mL of enclosed solvent and the DNA was then isolated from this suspension.

Isolation of viral DNA. The genetic material of CAV-2 was isolated by two methods: using a commercial genomic mini-DNA isolation kit (A&A Biotechnology, Gdańsk, Poland), and a manual method with a double phenol-chloroform extraction (Lantz et al., 1997). The performance of both methods was compared by measuring the isolated DNA concentration using the Picodrop UV/Vis spectrophotometer at a wavelength of 260 nm. The DNA isolated by both methods was assigned to the PCR.

Optimisation of the standart PCR for CAV-2. The optimisation included such factors as concentrations of magnesium ions ranging from 1.6 to 2.5 mM, dNTPs ranging from 50 to 200 µM, and primers ranging from 10 to 50 pM. The volume of reaction mixture was stable and amounted to 50 µl. In addition to the abovementioned reagents, it also contained Taq polymerase, each time in a concentration of 2.5 U and DNA (5 µl). Primers CAV-F 5'-GAGCAGGTAGTATGGAC-3' and CAV-R 5'-TCAGTAAAAGRAGCAAC-3' used in the reaction made it possible to amplify the E1B-19K gene fragment of 235 base pairs (Gerhold et al., 2007). Reactions were conducted using a Biometra thermocycler (Goettingen, Germany). The optimisation also covered the number of PCR cycles (35-45) and thermal conditions of individual reaction stages: denaturation at 94ºC for 60 s, hybridization of primers at 50–60ºC for 60 s, and strand extension at 70–75ºC for 60 s.

Electrophoresis. Products of the PCR were analysed using the electrophoresis method in 1.5% agarose gel, in a TBE buffer, at a voltage of 8 V/cm. Gels were stained with ethidium bromide. The size of products was determined according to the weight standard 100 bp DNA ladder (Fermentas, Lithuania).

Optimisation of the Real-Time PCR. The Real-Time PCR for all 20 samples of isolated DNA was conducted using a Corbett apparatus. The same pair of primers (CAV-F and CAV-R) was used, as per the traditional PCR method. PCR was conducted in real time, with a SYBR Green 1 dye, in thin-walled test tubes of 100 µl. The DyNAmo HS SYBR Green qPCR Kit (Finnzymes) was utilised for this method, enabling a highly specific reaction to be conducted.

The 20 µl reaction mixture consisted of: 2 µl of DNA template, 7.2 µl of water, 0.4 µl of each primer CAV-F and CAV-R (in a concentration from 10 to 50 pM), 10 µl of Master Mix (Finnzymes, Finland) containing the modified hot start Tbr polymerase (*Thermus brockianus*), buffer for Tbr polymerase, dNTP, $MgCl_2$ and the intercalating SYBR Green 1 dye.

As with the traditional PCR reaction, the optimisation covered the number of PCR cycles (35-60) and thermal conditions of individual reaction stages: denaturation at 94ºC for 60 s, hybridization of primers at 50–60ºC for 60 s, and strand extension at 70–75ºC for 60 s.

The fluorescence measurement of the reaction mixture and determination of the Ct value (the number of amplification cycles after which the fluorescence intensity of the resultant product is higher than the background fluorescence) were conducted in real time at the stage of complementary strand extension to the DNA template. To confirm the amplification specificity, the melting temperature (HRM) of PCR products was determined by a gradual increase in the reaction mixture temperature from 50 to 95°C, with the continuous measurement of fluorescence intensity. Additionally, after amplification, all Real-Time PCR products were analysed using the electrophoresis method in 1,5% agarose gel.

Sequencing. To evaluate the specificity of the primers annealing to the right genome sequence of CAV-2, ten randomly-selected products of the PCR and ten of the Real-Time PCR were purified (QIAquick PCR Purification Kit QIAGEN), sequenced, and then compared to the analogous sequence of the E1B-19K CAV-2 gene placed in the GenBank at no. AC 000003. The sequencing was conducted in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. The sequencing results were received via e-mail and developed using Lasergene DNASTAR software.

Results

DNA isolation. From the two methods of DNA isolation employed, the phenol method showed almost a three times higher performance than isolation using a commercial kit. The quantity of resultant DNA amounted to 98.8 ± 12.5 ng/µl, whereas the performance of the commercial method amounted to 34.2 ± 7.9 ng/µl.

PCR. The PCR was most efficient when the reaction mixture contained 2 mM of $MgCl_2$, 100 µM of dNTP and 50 pM of each primer, and its individual stages were run under the following conditions: denaturation at 94ºC for 60 s, hybridization of primers at 55ºC for 60 s, and strand extension at 72ºC for 60 s.

With such a composition of the reaction mixture, and under the abovementioned thermal conditions, the presence of viral DNA was revealed in all of the 20 examined samples (the PCR product was not obtained in the negative control). The size of resultant amplicons was 235 base pairs.

Real-Time PCR. In the Real-Time PCR, the genetic material of CAV-2 was also found in each of the 20 examined samples. The reaction was most efficient when the reaction mixture contained primers in a concentration of 50 pM, while the reaction conditions were as per the traditional PCR. In contrast to the traditional PCR, the amplification of CAV DNA in the Real-Time PCR was faster (the lowest Ct value was 13 cycles) (Fig. 1). The melting temperature for all obtained products was 83.5ºC (Fig. 2) which shows the specificity of primers annealing to the template, the lack of amplification of nonspecific products, and the formation of primer-dimer structures.

Sequencing results. The sequences obtained from 10 randomly-selected PCR and 10 randomly-selected Real-Time PCR products exhibited a mutual 100% homology of nucleotide sequences and a similarity of 99.6% with the standard CAV-2 AC 000003 sequence. The differences between amplicon sequences obtained in our studies and the standard sequence resulted from replacing cytosine with adenine at position 45 in our own strains of the virus with respect to the standard sequence.

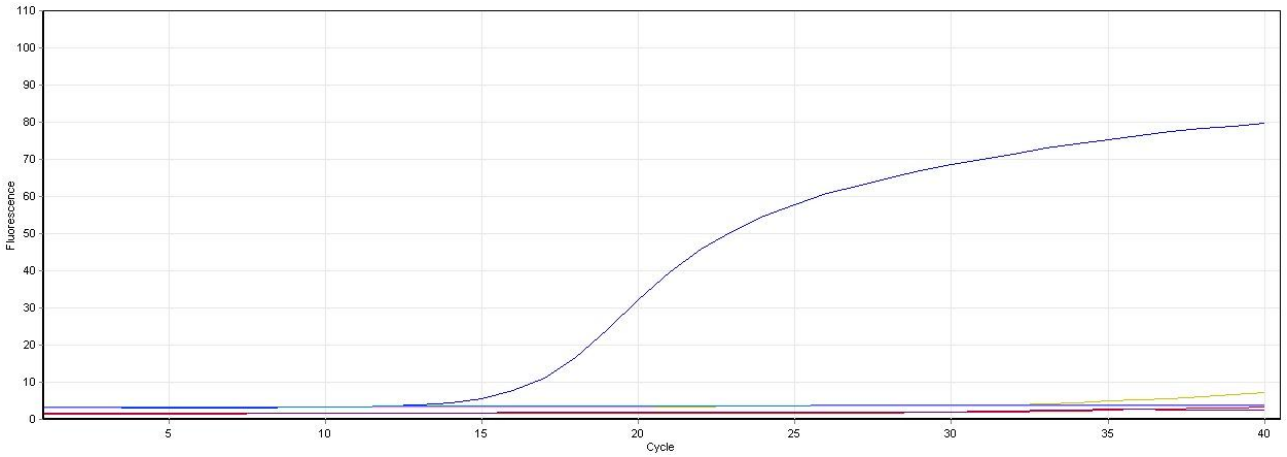

Fig.1. **Amplification curve. The lowest Ct value for the Real-Time PCR products of the E1B-19K gene of CAV-2 canis was about 13 cycles**

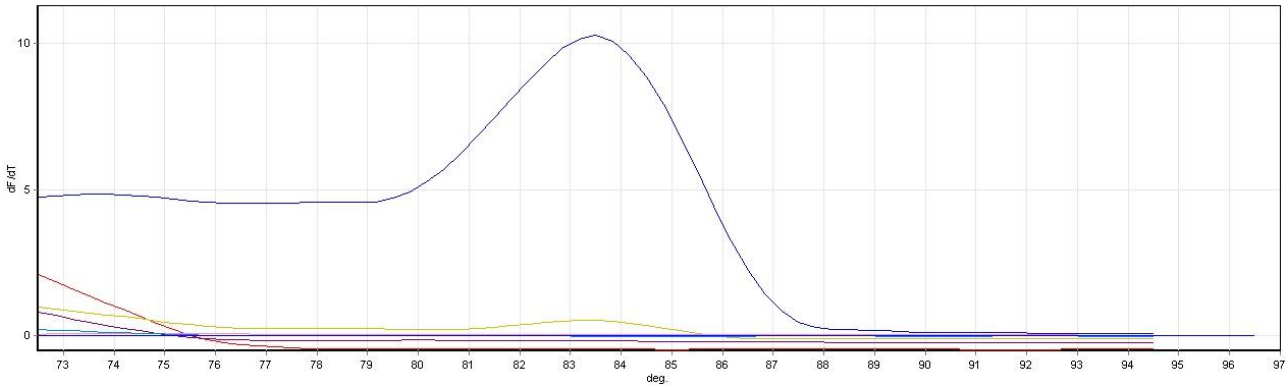

Fig .2. **The analysis of the CAV-2 amplicon melting curve obtained in the Real-Time PCR reaction**

Discussion and Conclusions

CAV-2 is one of the factors inducing canine infectious tracheobronchitis (kennel cough). The virus was isolated for the first time in 1961 in Canada from dogs with symptoms of upper respiratory tract infection (Ditchfield et al., 1962). Its presence was also revealed in dogs with symptoms of enteritis and in the brains of dogs exhibiting neurological symptoms (Benetka et al., 2006; Hamelin et al., 1985; Macartney et al., 1988).

The diagnostics of CAV-2 infections comprise techniques of different sensitivity and specificity. The most common are as follows: virus isolation in cell cultures, serological methods (ELISA, IF), immunohistochemical techniques and, recently, techniques of molecular biology (PCR, Real-Time PCR) (Greene, 2012; Mizak and Rzeżutka, 1998; Mizak and Rzeżutka, 1997). The methods of molecular biology enable detection of an aetiological factor for the disease and allow changes occurring in its genome to be followed, which are important from the point of view of virus taxonomy and disease immunoprophylaxis (Chaturved et al., 2008; Yoon et al., 2010).

In this study, optimisation, comparison and estimation of the usefulness of the PCR and Real-Time PCR techniques in the detection of CAV-2 infections was performed. The genetic material of the virus was isolated by two methods. Although the manual method is more time-consuming, it showed an almost three times higher performance than the commercial kit. This is important for the results of the PCR study, whose sensitivity depends to a high extent on the quantity of the template DNA used for amplification, which in turn depends on the method used to isolate the genetic material (Chesters, 1996; Lien and Lee, 2010; Miyachi, 2001). The predominance of the manual method of DNA isolation over commercially available kits is confirmed by the observations of Adaszek et al. (2012) who studied the impact of different techniques of nucleic acid isolation from blood on the detection effectiveness of subclinical infections caused by *Babesia canis* in dogs. The observations of these authors are similar to the results of our studies and indicate that the manual method with phenol and chloroform is the most effective for DNA isolation. When these authors used commercially available kits of low effectiveness for DNA isolation, the concentrations of amplicons obtained in the PCR were low and excluded the possibility of conducting further analysis (sequencing, digestion by restriction enzymes).

Positive results were obtained for all of the 20 DNA samples tested, both by the traditional method and by the Real-Time PCR. This indicates the high and comparable sensitivity of these methods. A suitable selection of the

base sequence, which is the template for nucleic acid multiplication, has an impact on the proper sensitivity and specificity of the PCR. Even a single point mutation in the amplified DNA, especially in the region of the third position of the primer, may cause falsely negative results for the reaction (Adaszek et al., 2009). It is possible to demonstrate the presence of variable regions in the template by sequencing (Kamińska and Dąbrowska, 2004). Thus, for more accurate analysis of the results obtained in the PCR reaction, the obtained amplicons were purified and sequenced. 10 randomly-selected and sequenced amplicons obtained in the traditional PCR and 10 in the Real-Time PCR exhibited a mutual 100% similarity and a similarity of 99.6% with the standard CAV-2 AC 000003 sequence, which proves the specificity of the PCR and the Real-Time PCR. Moreover, the specificity of the Real-Time PCR is supported by the melting temperature of the obtained amplicons, which was identical for all tested samples and amounted to 83.5ºC.

The Real-Time PCR proved to be less time-consuming than the standard PCR. By this method, it was possible to observe an increase of the amplified DNA after just 13 cycles, which enabled limitation of the number of PCR cycles and obtaining of the result after 30–35 minutes. Obtaining the study result in such a short time is extremely important for clinical practice (Heim et al., 2003; Wyczałkowska-Tomasik and Żegarska, 2009).

The high sensitivity and specificity of methods of molecular biology in diagnostics of adenoviral infections in dogs was proved by Benetka et al. (2006) who, using the PCR technique, demonstrated the presence of DNA CAV-2 in the brain of a dog with symptoms of central nervous system disorder. Other conducted tests, e.g. virus isolation in cell cultures and in-situ hybridisation, gave a negative result. In turn, the studies of Yoon et al. (2010) indicate that, although IHC and PCR methods have a similar sensitivity in detecting CAV-2 infections in dogs, only the first is considered to be basic in the diagnostics of adenovirosis. However, these authors recognised the PCR as complementary to the IHC. This suggests the need for further continuous improvement and optimisation of molecular biology techniques for detection of CAV-2 infections. This can be accomplished using the PCR reaction in real time (Real-Time PCR). Unquestionable benefits of this technique include a significantly higher sensitivity and specificity of the reaction due to the possibility of amplification of shorter products, and also a reduction of the contamination risk related to conducting a reaction in one test tube only (Stadejek, 2006). Currently, this method is widely used in the detection of viral, bacterial and parasitic infections, and also in oncology, in gene mutation and polymorphism detection tests, and in studies on medicines (Adaszek et al., 2011; Adaszek and Winiarczyk, 2010; Król et al., 2007; Kycko and Reichert, 2010; Wyczałkowska-Tomasik and Żegarska, 2009).

In conclusion, the techniques of molecular biology are becoming increasingly important in the laboratory diagnostics of CAV-2. As a result of properly conducted optimisation, these methods are sensitive and specific, and are characterised by high repeatability and good accuracy of the results. Due to these benefits, both the PCR and the Real-Time PCR are on target to become the standard diagnostic methods of CAV-2 infections.

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